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(54) Title: A MEANS AND A METHOD FOR IDENTIFICATION OF THE SEQUENCE OF DNA NUCLEOTIDES BY LASER SPECTROSCOPY

(57) Abstract

A means (DNA identification card) and a method for identification of the sequence of DNA nucleotides by laser spectroscopy. A DNA identification card is a solid means onto/into which the nucleotides or their groups cut off from the DNA molecule are carried and fixed with the same sequence as in the DNA and where the nucleotides or their groups are placed from one another at distances exceeding the wavelength of the light exciting the fluorescence of marker molecules or atoms. The nucleotides or their groups are identified and their sequences determined on the DNA identification card by the fluorescence spectrum of marked nucleotides using method of single molecular detection (SMD) or single molecular spectroscopy (SMS). High spectral resolution is achieved by cooling the DNA identification card to temperatures below 10 K and using specific markers which carry at these temperatures with them into the fluorescence excitation spectra narrow and intense zerophonon lines.

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TITLE

A means and a method for identification of the sequence of DNA nucleotides by laser spectroscopy

FIELD OF INVENTION

The present invention belongs to the field of molecular biology and laser spectroscopy, or , more specifically, to the field of identification of DNA primary structure by laser spectroscopic means.

BACKGROUND ART

Various chemical, enzyme and other methods, more recently also laser spectroscopic methods have been used for identification of DNA nucleotides and for establishing their sequence.

The closest solution to the means proposed by the present invention (the DNA identification card) appears to be a stream of liquid used in laser spectroscopy, in which DNA nucleotides are identified by detection at the single molecule level (SMD) (Peter M. Goodwin, Rhett L. Affleck, Patrick W. Abrose, James N. Demas, James H. Jett, John C. Martin, Linda J. Reha-Krantz, David J. Semin, Jay A Schecker, Ming Wu, Richard A. Keller,''Progress towards DNA Sequencing at the Single Molecule Level'', Proceedings of the International Workshop on Single Molecule Detection. Berlin, Germany, Oct. 4 - 6, 1995; Experimental Methods in Physics).

The method closest to the one described in the present invention is the laser spectroscopy method referred to above, which consists of the following stages. First of all the nucleotides in the DNA molecule are marked with molecules emitting characteristic fluorescence spectra - the so-called markers - so as to make it possible to differentiate between one to four out of four nucleotides A,C,G, and T. Secondly, the nucleotides are separated in succession one by one, using for this purpose chemical

methods or enzymes. Thirdly, the separated nucleotides are, one by one, and in the order of separation transported into a thin, precisely oriented jet of liquid, making sure that the distance between adjacent nucleotides within the stream is big enough to allow excitation of each single nucleotide separately by means of a laser beam, thus evoking its characteristic fluorescence. At the time one nucleotide is being excited, the other nucleotides are not subjected to excitation. In the fourth stage of the method, the nucleotides within the jet are identified and their sequence established via their markers' characteristic fluorescence at the level of single molecule detection (SMD). SMD will reveal, which nucleotide within the jet passed through the focus of the laser beam. In a similar fashion the same is found about all the nucleotide sequences within the DNA molecule. The method has the following shortcomings. First, the time T for identifying the nucleotides is rather short, the molecule passing through the diameter d of the laser beam' s focus, which approximately equals 10^{-3} cm, within the time T= d : v, where

T - denotes the time within which the nucleotide passes through the laser beam' s focus;

d - the diameter of the laser beam' s focus;

v - the velocity of the molecule.

Thus, when the velocity v of the molecule is 1 cm/ s^{-1}, then T equals 10^{-3} s.

In case the measuring procedure lasts longer, the speed and efficiency of the method are lower, i.e. the number of nucleotides identified per second is smaller. Secondly, even slight ruffling of the surface of the stream of liquid may cause the marked nucleotides to miss the laser focus and some nucleotides to pass unnoticed.

A considerable shortcoming of the method used for measuring within the stream of liquid is the fact that the stream disappears immediately after the measurement, thus excluding the possibility of repeating the measurement procedure of the nucleotide sequence of the same DNA molecule, in order to verify the initial result.

TECHNICAL PROBLEM

In case the DNA nucleotide sequences are identified by means of laser spectroscopy, the question is, how to guarantee high sensitivity and selectivity of spectroscopic detection, and make it possible to repeat the measurement procedure. The fact that the distance at which nucleotides are placed in DNA, 3.4 Å, is a thousand times smaller than the wavelength of light, excludes the possibility of immediate selectivity in space, i.e., of finding the location of nucleotides within the DNA molecule by means of optical methods suited for measuring within the visible light range.

THE SOLUTION

In order to solve the above problems, the present invention proposes a means and a method, which will guarantee high sensitivity and selectivity of spectroscopic detection and will allow the measuring procedure to be repeated.

The invention proposes a DNA identification card (IC)to be used, in which the sequence of nucleotides coincides with their sequence in DNA, and a method, in which the fluorescence spectra of the marker molecules or atoms carried on to the IC are measured by way of SMD or SMS.

DISCLOSURE OF INVENTION

The aim of the present invention is to propose a means and a method for identification of nucleotide sequences within the DNA molecule by means of spectroscopy and high resolution laser spectroscopy, in which single nucleotides or groups

of nucleotides which have been cut off from the end of a DNA molecule are transported by means of a jet of gas, or a continous stream of liquid, a stream composed of drops of liquid onto/into a solid surface in this way that the sequence of nucleotides remains identical with that within the DNA molecule, but the intermolecular distances between the adjacent nucleotides are larger than the wavelength of light by which the fluorescence of the marker molecules or atoms is exited. For this purpose, the solid means is moved along a linear or spiral or some other trajectory against to the stream at a suitable speed (ca 1 cm $/s^{-1}$). As a result of that, the solid together with the nucleotides of fixed structure on/in it will form an identification card, which retains the sequence of nucleotides within the DNA molecule. The IC will be the means for identifying the nucleotide sequence within DNA by laser spectroscopy.

In the first embodiment of the said method, the DNA nucleotides or their groups to be cut off the molecule are marked either before or after they are cut off the DNA molecule, or after they have been carried onto/into the surface of the IC by means of marker molecules or atoms having a characteristic spectra of fluorescence, and the nucleotides are identified and their sequence determined on/in the surface of the IC by laser optical SMD , either at room temperature or at lower temperatures.

In the second embodiment of the method, the IC is cooled down to reach temperatures below 10 K and the nucleotides are identified on/in its surface by SMS of the solid´s zero-phonon lines(ZPL), whereas the nucleotides or groups of nucleotides to be severed from the DNA molecule are marked with such marker-molecules or atoms which at temperatures below 10 K have in their fluorescence spectra an intense and narrow purely electronic zero-phonon line.

The advantage of the second embodiment of the method, as compared to the prototype method of single molecule laser spectroscopic detection (SMD) in a jet of liquid, consists

in that spectral selectivity of nucleotide sequence identification is raised by 4-5 orders of magnitude.

DESCRIPTION OF DRAWINGS

The drawings describe the intensity of fluorescence of the nucleotides, as dependent on the frequency of exitation, to illustrate the second version of the second embodiment.

Fig. 1 - frequency of excitation ω_{L1}

Fig. 2 - frequency of excitation ω_{L2}

Fig. 2 - frequency of excitation ω_{L3}

In the drawings, the diameter of the circles denotes intensity of fluorescence of one single nucleotide at the given frequency of excitation ω_{L1} (Fig. 1). With the frequency changing within the range of ZPL, absorption will change and accordingly (according to the ZPL contour) the fluorescence emitted by the given molecule will either grow or diminish. In Fig. 2 the frequency ω_{L2} is closer to the peak value of ZPL than in case of ω_{L1} for nucleotides No 1 and 2, and farther for nucleotides No 3 and 4; ω_{L2} happens to be at the beginning of the ZPL absorption contour for a new nucleotide No 5.

$\omega_{L2} = \omega_{L1} + \Delta\omega; \quad \Delta\omega < \delta_{ZPL}$

$\Delta\omega$ - a slight change of excitation frequency in scanning;

δ_{ZPL} - the width of the zero-phonon line.

In Fig. 3 at the frequency ω_{L3} a different set of nucleotides gets excited, the frequency of excitation having been changed well over the ZPL width δ_{ZPL} .

$$\omega_{L1} = \omega_{L1} + \Delta\omega_3 \; ; \quad \Delta\omega_3 >> \delta_{ZPL}$$

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention offers a means described below for determining the sequence of DNA nucleotides by laser spectroscopy. The said means (DNA identification card) is a solid means onto/into which the nucleotides or their groups cut off from the DNA molecule are carried and where the nucleotides or their groups are placed from one another at distances exceeding the wavelength of light exciting the fluorescence of marker molecules or atoms, the nucleotides or their groups are marked before or after carrying with marker molecules or atoms forming the DNA identification card in which the nucleotide sequence corresponds to their sequence in DNA.

To determine the nucleotide sequence the fluorescence spectra of the marker molecules/atoms are measured on the solid means (DNA identification card) by the single molecule detection (SMD) or single impurity molecule spectroscopy (SMS) method.

As the solid means, for example, a plate or tape of polymer or paper can be used which is moved linearly or spirally or at some other suitable trajectory with respect to the liquid or gas stream at such speed (ca 1 cm s^{-1}) that the nucleotides or their groups remain at distances exceeding the wavelength of light exciting the fluorescence of marker molecules or atoms. The nucleotide sequence in the liquid or gas stream is transferred to the spatial nucleotide sequence onto/into the identification card while the distances between the nucleotides are bigger than the wavelength of

light exciting the fluorescence of marker molecules or atoms. In such a way a fixed nucleotide structure is formed onto/into solid means which is called the identification card. In the sense of the present invention, the DNA identification card is a solid means with nucleotides carried the onto/into it, the sequence of the nucleotides being identical with that of the DNA molecule and from which the fluorescence spectra of the marker molecules/atoms are measured by the single molecule detection (SMD) or single impurity molecule spectroscopy (SMS) method.

The nucleotides or their groups cut off from the DNA molecule can be marked, additionally marked or re-marked on the identification card as well.

If the nucleotides are carried onto the identification card perpendicularly to the direction of the movement of the identification card with deviations r, where r is bigger than the wavelength λ, the second version of the second application (SMS) described below is used which enables simultaneous detection of the nucleotide strip with width up to 10^{-1} cm (cf. Fig. 1-3).

The DNA identification card obtained by appliction of the invention is a record file which can be repeatedly investigated from various aspects, and by using different methods, the results obtained in different laboratories can be measured again and compared. For example, it is possible to determine the higher-order correlations in the nucleotides location, to use coherent optical methods, among them holography of various kinds. It is possible to create data banks of natural samples of DNA molecules. Very high spectral resolution allows us to identify not only individual nucleotides, but also their n-membered sets where n can be tens. Therefore it is not indispensable that single nucleotides would be cut one by one.

In principle only one DNA molecule is sufficient to get one identification card, from two DNA molecules we get two identification cards whose collation allows error correction and enables complementary aspects of investigation. The DNA identification card of a testee (patient) can be compared later, even years after, with the freshly prepared DNA identification card of the same tested organism. The inconsiderable ageing of the identification card at low temperatures due to the diffusion and spectral diffusion can be compensated for very high precision of the above-mentioned SMS method. The identification card can be measured and investigated at room temperatures, i.e. at ca 300 K by the SMD method similarly to the one described in the prototype method made in Prof. Keller's laboratory. At low temperatures (below 77 K) a better spectral resolution and identification card preservation is achieved. When measuring the identification card at temperatures below 10 K by the SMS solid state zero-phonon lines method the spectral selectivity increases 4-5 times in comparison with SMD method (detection in a stream of liquid without the identification card or at room temperature on the identification card) used in the prototype.

The difference and advantages are that using the identification card makes use of different methods and repeated measurements possible, also in different laboratories.

The method has two embodiments which consist of the following stages.

The first embodiment is the single molecule detection (SMD) on the level where spectral selectivity is not high.

The parts of the DNA molecule to be severed will be marked with marker molecules/atoms having characteristic fluorescence spectra either before or after they are cut off

from DNA. The same known markers are used, for example those which have been used at Prof. Keller's laboratory.

Secondly, the nucleotides or their groups are cut off one by one from the end of the DNA molecule, for example by use of enzymes. The cutting off and marking, incl. re-marking of the parts of the DNA molecule takes place at a suitable temperature, for example at room temperature. Higher temperature will essentially accelerate the cutting procedure - the temperature rise by 1° C will accelerate it up to 6 times.

Thirdly, the nucleotides or their groups which have been cut off are carried into the gas or liquid stream (drops) and transported with that onto/into the solid means (identification card). In the next stage of the method the nucleotides are identified and their sequence is established with laser optics by the SMD method and level. The identification card measuring can occure at room temperature (300 K) or at lower temperatures. Spectral selectivity at room temperature is not high, therefore the variant where the identification card is cooled down to temperature below 77 K is used, which guarantees higher spectral selectivity and a better preservation of identification cards.

The other embodiment of the method based on the zero-phonon lines (ZPL) spectroscopy guarantees very high spectral selectivity when measuring on the identification card, and thanks to high-precision measurements of the spectra the reliability of results is increased essentially. The difference from the first embodiment consists in the fact that the identification card is cooled down to 10 K and suitable markers with zero-phonon lines are selected. According to this embodiment, the parts to be cut off from the DNA molecule are marked before or after they are cut off from the DNA with such marker molecules/atoms which at temperatures below 10 K have intense and narrow zero-phonon

lines fluorescence spectra. Markers containing for example the ions of rare earth metals or organic molecules with porphyrine core are used as promoters of ZPL. Secondly, the nucleotides or their groups are cut off one by one from the end of the DNA molecule, using, for example, enzymes. The severing and marking, incl. re-marking, of the parts of the DNA molecule takes place at a suitable temperature, for example at the room temperature. The marking and re-marking can proceed either at room temperature or at higher or lower temperatures. The peak value of the ZPL absorption cross-section increases at low temperatures considerably (up to 4-5 orders of magnitude). Below 10 K thousands of impurity molecules and atoms are suitable for the present method, i.e. they have sharp (with small half-width) ZPL with high peak intensity (the peak value for absorption cross-section from 10^{-10} to 10^{-11} cm^{-2}) in many (thousands of) matrices, which demonstrates the potential this method has for finding further suitable markers.

In case of the embodiment described here, the indicator of sensitivity and selectivity for spectroscopic detection - the absorption cross-section σ_0 for ZPL - is for example at 2 K ca 10^5 times bigger than the σ_0 at room temperature. Low temperature increases the ZPL absorption cross-section $\sigma_0(T)$ 10^5 times in comparison with room temperature:

$$\sigma_0(T = 2\ K) : \sigma_0(T = 300\ K) = 10^5.$$

In the fifth stage the nucleotides (or their groups of 10-100) are identified and their sequence is determined by the SMS methods and level by means of ZPL laser optics. It is essential to note that the availability of ZPL allows us to use not only the fluorescence excitation but also coherent methods of optics, such as holography, in identification card measurements.

For fluorescence excitation with high spectral selectivity, the dye laser of a narrow (laser linewidth 1-2 Mhz) and stable frequency is used, for example Coherent Radiation Single Mode Dye Laser CR-699-29 which is pumped for example with an argon laser. For fluorescence photon counting, known systems with a photomultiplier as their base element are used (in this case FEV-79 whose quantum yield is up to 12% at the wavelength 633 nm); the signal is sampled with a multichannel analyzer (in the present case LP-4900). Additionally, measurement automation hard- and software were used.

The second embodiment of the present invention uses two versions of single impurity molecule spectroscopy (SMS) of solid state zero-phonon lines (ZPL) for exciting fluorescence of marked nucleotides. The nucleotide sequence can be identified not only along the one-dimensional line determined by a sharp laser focus (whith the focus diameter d within the range of 1 to 10 λ) whose width is d, but also along a remarkably wider strip D, where D equals roughly from 100 to 1000 μm. Therefore the ruffling of the liquid stream will not interfere with the applicability of the present method.

In the case of both variants it is necessary to scan the exciting frequency over the zero-phonon lines spectrum. The difference consists in the spatial distribution of laser radiation and consequently in the field of vision.

In the second embodiment of the present method, corresponding to the first, basic version of the SMS, the exciting light spot is sharply focused (i.e. reaching up to the diameter d which is close to the diffraction limit wavelength λ, where d equals λ or is a few times bigger). This mode proves troublesome if the width of the nucleotides distribution strip r on the identification card is many

times bigger than λ and therefore renders necessary high precision scanning of one and the same section of spatial distribution many times (tens and hundreds of times). According to the second application of the same embodiment excitation is performed by a nonsharply focused laser beam, where the focus diameter d exceeds the diffraction limit λ tens or hundreds of times. In this way, we achieve the simultaneous excitation of all the marked nucleotides on the identification card within approximately 1 mm^2. The nucleotides are identified within a 0.1-1 mm wide strip of the identification card by moving the identification card against the laser beam and one scan (or a few scans) along the stream track on the identification card is sufficient. The precision of laser frequency scanning exceeds the ZPL width. Owing to the intensity dependence of fluorescence, microscopy will provide a frequency-and-space-domain view of all the marked nucleotides within the range approximately equal to D^2 (cf. Fig. 1-3).

Now the abovementioned second excitation mode is used in the case when D is approximately equal to 100 μm, but it is possible to achieve a tenfold, i.e. when D is approximately equal to 1 mm. Therefore the proposed SMS embodiment makes it possible to compensate for the identification card deviations from the given trajectory axis within the range of up to 1 mm by means of one spatial scan.

The presnt invention allows for spatial and spectral viewing and simultaneous very high spectral selectivity identification of lots of nucleotides or their groups located on the identification card within dimensions 0.01 - 1 mm^2. The invention will both raise the speed of measuring and enhance its reliability.

CLAIMS

1. A means for determining the sequence of DNA nucleotides by laser spectroscopy is a solid means (DNA identification card) onto/into which are carried the nucleotides or their groups cut off from the DNA molecule and marked before or after they are cut off with such marker molecules/atoms which have characteristic fluorescence spectra, whereas the nucleotides or their groups are placed on/in the solid means at such distances from one another which exceeds the wavelength of light exciting the fluorescence of marker molecules/atoms, and the nucleotide sequence on/in which is determined via the fluorescence spectra of marker molecules/atoms, using for this purpose single molecule detection or single impurity molecule spectroscopy.

2. A method for determining the sequence of DNA nucleotides by laser spectroscopy, where the nucleotides to be cut off from the DNA molecule are marked with marker molecules/atoms, then cut off from the DNA molecule and transferred into a liquid stream and the nucleotides are identified via the fluorescence spectra of the said marker molecules/atoms, c h a r a c t e r i z e d i n this that the nucleotides or their groups cut off from the DNA molecule are transported with the liquid stream or drops onto/into a solid means, and the said solid means is simultaneously moving at such speed that the nucleotides or their groups located on/in the solid means are separated from one another at distances exceeding the wavelength of light exciting the fluorescence of the marker molecules/atoms, and the solid means together with the nucleotides or their groups carried onto/into it forms the DNA identification card, and the nucleotides on/in it are identified and their sequence determined via the fluorescence spectra of the marker molecules/atoms by means of single molecule detection or single impurity molecule spectroscopy.

3. A method according to claim 2 characterized in this that the nucleotides cut off from the DNA molecule are marked with such marker molecules/atoms which at temperatures below 10 K have intense and sharp ZPL fluorescence spectra, the said solid means (DNA identification card) is cooled down to temperatures below 10 K and the nucleotides or their groups on/in the solid means (DNA identification card) are identified and their sequence is determined via the fluorescence spectra of the marker molecules/atoms, using solid state laser spectroscopy of zero-phonon lines.

4. A method according to claims 2 or 3 characterized in this that the nucleotides cut off from the DNA molecule are marked with marker molecules/atoms after they have been cut off from the DNA molecule.

5. A method according to claims 2, 3 or 4 characterized in this that the laser beam is sharply focused on the DNA identification card.

6. A method according to claims 2, 3 or 4 characterized in this that the laser beam is nonsharply focused on the DNA identification card.

7. A method according to claims 2, 3,4, 5 or 6 characterized in this that instead of a liquid stream or drops a gas stream is used.

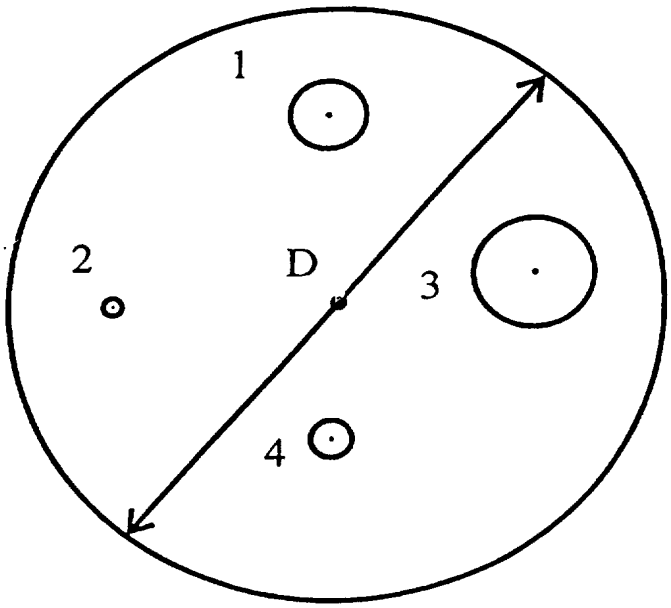

Fig. 1

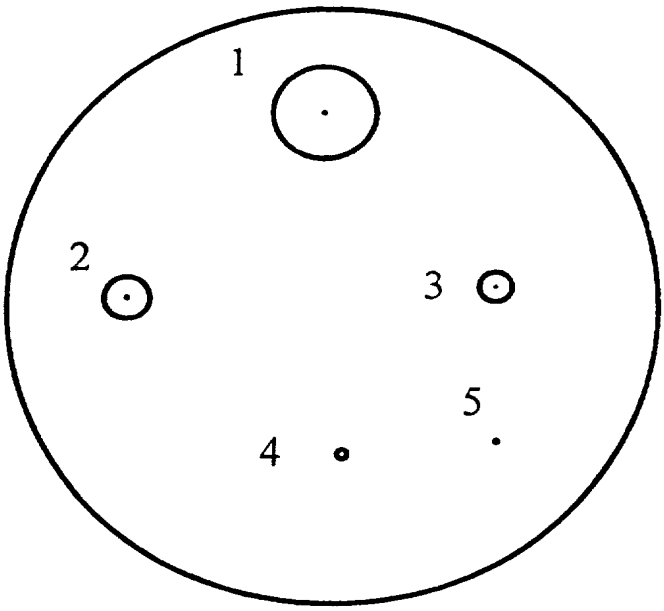

Fig. 2

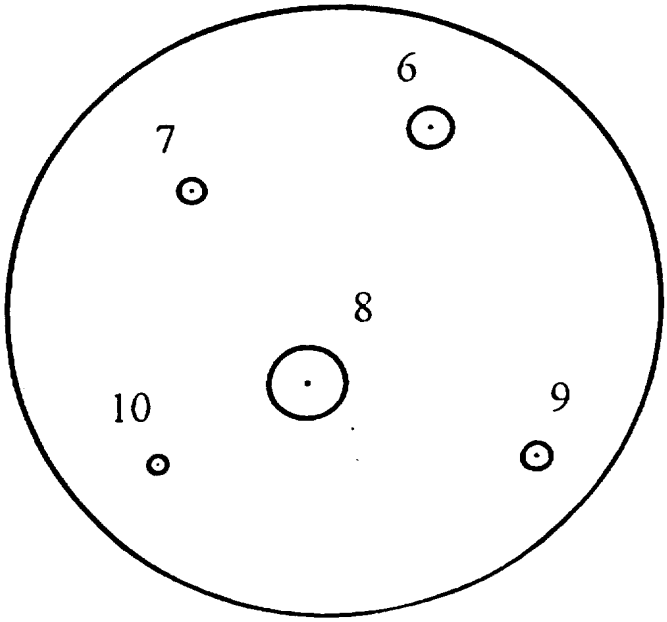

Fig. 3

INTERNATIONAL SEARCH REPORT

Inter nal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/00 G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 18218 A (SEQ LTD) 18 August 1994 see the whole document ---	1-7
Y	WO 96 24689 A (SARGENT JEANNINE P ;MARKS JEFF (US)) 15 August 1996 see the whole document ---	1-7
A	HARDING AND KELLER: "SINGLE-MOLECULE DETECTION AS AN APPROACH TO RAPID DNA SEQUENCING" TIBTECH, vol. 10, 1992, pages 55-57, XP002052227 see the whole document --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
15 January 1998	28/01/1998

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EE 97/00003

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DAVIS L M ET AL: "RAPID DNA SEQUENCING BASED UPON SINGLE MOLECULE DETECTION" GENETIC ANALYSIS TECHNIQUES AND APPLICATIONS, vol. 8, no. 1, 1991, pages 1-7, XP002039495 see the whole document	1-7

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EE 97/00003

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9418218 A	18-08-94	AU 1228197 A	27-03-97
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